MOLECULAR SIGNATURE AND ASSAY FOR FLUOROQUINOLINE RESISTANCE IN *BACILLUS ANTHRACIS*

CLAIM TO DOMESTIC PRIORITY

[00001] This application claims priority to U.S. Provisional application Serial No. 60/417,843 entitled "Molecular Signature and Assay for Fluoroquinoline Resistance in *Bacillus Anthracis*" filed October 11, 2002, by Paul S. Keim *et al.*, and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[00002] This invention concerns generally molecular assay for *Bacillus anthracis* strains and more particularly, primers, methods and kits for identifying fluoroquinolone resistance in *Bacillus anthracis.*

BACKGROUND OF THE INVENTION

[00003] *Bacillus anthracis (B. anthracis)* regularly infects livestock and wild ungulates, causing the disease anthrax. Although globally dispersed and endemic to many regions, *B. anthracis* shows little genetic variation between strains. Population studies using various methods of analysis including Pulse Field Gel Electrophoresis (PFGE) (Harrell et al., 1995), Single Nucleotide Polymorphisms (SNP) (Price et al., 1999; Harrell et al., 1995), Amplified Fragment Length Polymorphisms (AFLP) (Keim et al., 2000) have all found *B. anthracis* to be highly monomorphic.

[00004] A spore-forming zoonotic, *B. anthracis* occasionally infects humans, causing cutaneous, intestinal or pulmonary forms of anthrax (Friedlander, 1999). Although all three human forms are rare, the potential for using *B. anthracis* as a

biological weapon makes development of antibiotic resistance a particularly relevant concern.

[00005] The preferred therapeutics for human anthrax infections is the fluoroquinolone, ciprofloxacin (CIP). Fluoroquinolone bactericidal action is on gyrase-DNA and topoisomerase IV-DNA complexes where drug binding causes the release of double-stranded DNA breaks (Drlica and Zhao, 1997, Piddock, 1999). Fluoroquinolone resistant mutants have amino acid changes in Quinolone Resistance Determining Regions (QRDRs) of the GyrA subunit of gyrase and the ParC subunit of topoisomerase IV. Resistance can also arise from the over-expression of multi-drug efflux pumps of the major facilitator superfamily. Low-level resistance can be acquired with a single missense mutation within a QRDR or a point mutation. in the regulatory region of an efflux pump. However, high-level resistance requires a combination of mutations. The stepwise accumulation of QRDR mutations required for high-level resistance appears to follow a species-specific and predictable pathway (Ng, et al., 1996; Ferrero, et al., 1995).

[00006] Fluoroquinolone resistance, like resistance to many other antibiotics, is becoming prevalent in several clinically important species due largely to non-compliance with recommended fluoroquinolone regimens and standard regimens that are insufficient for producing inhibitory concentrations of fluoroquinolones in the soft tissue of patients (Brunner, et al., 1999). In addition to clinical sources, the use of fluoroquinolones in food-animal production has been identified as a major contributor to the emergence of fluoroquinolone resistance (Endtz et al., 1990; van den Bogaard, et al., 2000; van den Bogaard, et al., 2001).

[00007] Culturing an anthrax sample and exposing the culture to fluoroquinolene has previously been the method used to determine whether a particular strain of

anthrax exhibits fluoroquinolene resistance. However, growing a bacterial culture requires a critical mass of bacterial cells in order for the culture to grow. Further, culturing is not rapid in that the bacterial culture must grow to a visible size in order to determine whether the strain is resistant.

[00008] Thus, a need exists for a means and method of reliably and rapidly determining fluoroquinolone resistant strains of *B. anthracis* from a small sample in order to ràpidly diagnosis anthrax and develop therapeutic methods of treating anthrax, especially in developing effective tools for use in detecting and treating resistant strains of anthrax that may be used in a bioterrorism attack. Additionally, a rapid molecular-based detection method is needed to perform epidemiological studies of anthrax infections.

SUMMARY OF THE INVENTION

[00009] It has been discovered that single nucleotide changes associated with fluoroquinoline resistance in *B. anthracis* mutants provide the basis of a rapid assay for detecting fluoroquinoline resistant, species directly from *B. anthracis* DNA. Cipro-resistant mutants have been isolated and characteristic SNP sequences have been identified. Primers and primer pairs are presented for assaying these SNP sequences in amplification assays, preferably multiplex. Kits useful for multiplexing sample DNA from *B. anthracis* strains are given.

[00010] Isolated oligonucleotides are provided comprising at least 12 consecutive nucleotides of a nucleic acid sequence selected from the group of consisting of: SEQ ID NO: 1; SEQ ID NO: 2; SEQ ID NO:3; SEQ ID NO: 4; SEQ ID NO: 5; SEQ ID NO: 6; SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16; SEQ ID NO: 17; SEQ ID NO: 18 SEQ ID NO: 19; SEQ ID NO: 20; SEQ ID

NO: 21; SEQ ID NO: 22; SEQ ID NO: 23; SEQ ID NO: 24; SEQ ID NO: 25; SEQ ID NO: 26; SEQ ID NO: 27; SEQ ID NO: 28; SEQ ID NO: 29; SEQ ID NO: 30; SEQ ID NO: 31; SEQ ID NO: 32; SEQ ID NO: 33; SEQ ID NO: 34; SEQ ID NO: 35; SEQ ID NO: 36; SEQ ID NO: 37; SEQ ID NO: 38; SEQ ID NO: 39; SEQ ID NO: 40; SEQ ID NO: 41; SEQ ID NO: 42; SEQ ID NO:43; SEQ ID NO: 44; SEQ ID NO: 45; SEQ ID NO: 46; SEQ ID NO: 47; SEQ ID NO: 48; SEQ ID NO: 49; SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; and SEQ ID NO: 53; wherein the oligonucleotide is capable of binding selectively to DNA indicating fluoroquinoline resistance in *Bacillus anthracis*.

[00011] In certain preferred embodiments of the invention the oligonucleotide are immobilized on a solid surface, a chromatographic surface, e.g., or a nanometric scale diagnostic plate. In other preferred embodiments of the invention the nucleotides further comprise an observable marker, most preferably a fluorescent label or a radioactive group.

[00012] Primer pairs selected from the group of oligonucleotide pairs consisting of: SEQ ID NO: 1 and SEQ ID NO: 2; SEQ ID NO: 3 and SEQ ID NO: 4; SEQ ID NO: 5 and SEQ ID NO: 6; SEQ ID NO: 7 and SEQ ID NO: 8; SEQ ID NO: 9 and SEQ ID NO: 10; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; SEQ ID NO: 15 and SEQ ID NO: 16; SEQ ID NO: 17 and SEQ ID NO: 18; SEQ ID NO: 19 and SEQ ID NO: 20; SEQ ID NO: 21 and SEQ ID NO: 22; SEQ ID NO: 23 and SEQ ID NO: 24; SEQ ID NO: 25 and SEQ ID NO: 26; SEQ ID NO: 27 and SEQ ID NO: 28; SEQ ID NO: 29 and SEQ ID NO: 30; SEQ ID NO: 31 and SEQ ID NO: 32; SEQ ID NO: 33 and SEQ ID NO: 34; SEQ ID NO: 35 and SEQ ID NO: 36; SEQ ID NO: 37 and SEQ ID NO: 38; and SEQ ID NO: 39 and SEQ ID NO: 40 are

presented wherein the pair of oligonucleotide primers is capable of binding selectively to DNA indicating fluoroquinoline resistance in *Bacillus anthracis*.

[00013] Internal oligonucleotide primer selected from the group consisting of: SEQ ID NO: 41; SEQ ID NO: 42; SEQ ID NO:43; SEQ ID NO: 44; SEQ ID NO: 45; SEQ ID NO: 46; SEQ ID NO: 47; SEQ ID NO: 48; SEQ ID NO: 49; SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; and SEQ ID NO: 53 are presented wherein the primer is capable of detecting a single nucleotide polymorphism, wherein the single nucleotide polymorphism is characteristic of fluoroquinoline resistance in *Bacillus anthracis*.

[00014] Single base extension (SBE) primers comprising the internal oligonucleotide primers and a polypolynucleotide tails for use in the amplification and separation of SNP in a PCR instrument wherein the SBE primers provide customizied amplicon lengths to aid electrophoretic separation of the amplicons.

[00015] In an important aspect of the invention methods are presented for detecting fluoroquinolone resistant *B. anthracis* strains by detecting the presence or absence of a plurality of selected target DNA sequences associated with fluoroquinolone resistance in *Bacillus anthracis*.

[00016] Certain preferred methods for detecting a fluoroquinoline resistant strain of *Bacillus anthracis* comprise the steps of:

i. providing a DNA sample from a *Bacillus anthracis* strain;

ii. providing one or more primer pairs selected from the group of oligonucleotide pairs consisting of: SEQ ID NO: 1 and SEQ ID NO: 2; SEQ ID NO:3 and SEQ ID NO: 4; SEQ ID NO: 5 and SEQ ID NO: 6; SEQ ID NO: 7 and SEQ ID NO: 8; SEQ ID NO: 9 and SEQ ID NO: 10; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; SEQ ID NO: 15

and SEQ ID NO: 16; SEQ ID NO: 17 and SEQ ID NO: 18; SEQ ID NO: 19 and SEQ ID NO: 20; SEQ ID NO: 21 and SEQ ID NO: 22; SEQ ID NO: 23 and SEQ ID NO: 24; SEQ ID NO: 25 and SEQ ID NO: 26; SEQ ID NO: 27 and SEQ ID NO: 28; SEQ ID NO: 29 and SEQ ID NO: 30; SEQ ID NO: 31 and SEQ ID NO: 32; SEQ ID NO: 33 and SEQ ID NO: 34; SEQ ID NO: 35 and SEQ ID NO: 36; SEQ ID NO: 37 and SEQ ID NO: 38; and SEQ ID NO: 39 and SEQ ID NO: 40; wherein the pair of oligonucleotide primers is capable of binding selectively to DNA indicating fluoroquinoline resistance in *Bacillus anthracis*;

iii. amplifying said DNA with one ore more said primer pairs; and

iv. comparing the results of said multiplexing step with results of amplification of DNA from known fluoroquinoline resistant strains.

[00017] Preferrably amplification of DNA is by multiplexing with one or more suitable primer pairs. Other preferred embodiments of the method for detecting fluoroquinoline resistance in *Bacillus anthracis* comprises the steps of:

i. providing a DNA sample from *Bacillus anthracis*;

ii. providing one or more isolated oligonucleotide comprising at least 12 consecutive nucleotides of a nucleic acid sequence selected from the group of consisting of: SEQ ID NO: 1; SEQ ID NO: 2; SEQ ID NO:3; SEQ ID NO: 4; SEQ ID NO: 5; SEQ ID NO: 6; SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16; SEQ ID NO: 17; SEQ ID NO: 18, SEQ ID NO: 19; SEQ ID NO: 20; SEQ ID NO: 21; SEQ ID NO: 22; SEQ ID NO: 23; SEQ ID NO: 24; SEQ ID NO: 25; SEQ ID NO: 26; SEQ ID NO: 27; SEQ ID NO: 28; SEQ ID NO: 29; SEQ ID NO: 30; SEQ ID NO: 31; SEQ

ID NO: 32; SEQ ID NO: 33; SEQ ID NO: 34; SEQ ID NO: 35; SEQ ID NO: 36; SEQ ID NO: 37; SEQ ID NO: 38; SEQ ID NO: 39; SEQ ID NO: 40 SEQ ID NO: 41; SEQ ID NO: 42; SEQ ID NO:43; SEQ ID NO: 44; SEQ ID NO: 45; SEQ ID NO: 46; SEQ ID NO: 47; SEQ ID NO: 48; SEQ ID NO: 49; SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; and SEQ ID NO: 53; wherein the oligonucleotide is capable of binding selectively to DNA indicating fluoroquinoline resistance in *Bacillus anthracis*.

iii. combining said oligonucleotides and said DNA under conditions whereby said DNA binds to said oligonucleotides; and

iv. detecting the presence or absence of bound oligonucleotides;

wherein the presence of bound oligonucleotide indicates a fluoroquinoline resistant *B. anthracis* strain.

[00018] Preferably in this preferred method the oligonucleotides comprise an observable marker, most preferably fluorescent or radioactive group. Other preferred methods of the present invention for detecting a fluoroquinoline resistant strain of *Bacillus anthracis* comprise the steps of:

i. providing a DNA sample from a *Bacillus anthracis* strain;

ii. providing one or more primer pairs selected from:

a) A pair of oligonucleotide primers selected from the group of oligonucleotide pairs consisting of: SEQ ID NO: 1 and SEQ ID NO: 2; SEQ ID NO:3 and SEQ ID NO: 4; SEQ ID NO: 5 and SEQ ID NO: 6; SEQ ID NO: 7 and SEQ ID NO: 8; SEQ ID NO: 9 and SEQ ID NO: 10; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; SEQ ID NO: 15 and SEQ ID NO: 16; SEQ ID NO: 17 and SEQ ID NO: 18; SEQ ID NO: 19 and SEQ ID NO: 20; SEQ ID NO: 21 and SEQ ID NO: 22; SEQ ID NO: 23 and SEQ ID NO: 24; SEQ ID

NO: 25 and SEQ ID NO: 26; SEQ ID NO: 27 and SEQ ID NO: 28; SEQ ID NO: 29 and SEQ ID NO: 30; SEQ ID NO: 31 and SEQ ID NO: 32; SEQ ID NO: 33 and SEQ ID NO: 34; SEQ ID NO: 35 and SEQ ID NO: 36; SEQ ID NO: 37 and SEQ ID NO: 38; and SEQ ID NO: 39 and SEQ ID NO: 40; wherein the pair of oligonucleotide primers is capable of binding selectively to DNA indicating fluoroquinoline resistance in *Bacillus anthracis;*

b) an internal oligonucleotide primer selected from the group consisting of: SEQ ID NO: 41; SEQ ID NO: 42; SEQ ID NO:43; SEQ ID NO: 44; SEQ ID NO: 45; SEQ ID NO: 46; SEQ ID NO: 47; SEQ ID NO: 48; SEQ ID NO: 49; SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; and SEQ ID NO: 53, wherein the primer is capable of detecting a single nucleotide polymorphism, wherein the single nucleotide polymorphism is characteristic of fluoroquinoline resistance in *Bacillus anthracis*; or

c) combinations thereof ;

iii. amplifying said DNA with one or more said primers, said primers preferably comprising an observable marker, most preferably a fluorescent or radioactive group; and

iv. comparing the results of said amplificatin step with results of amplification of a known fluoroquinoline resistant *B. anthracis* strain with said primers.

[00019] In preferred medhods, single base extension (SBE) primers (Table 3) comprising an internal oligonucleotide primer and further comprising polynucleotide tails for use in the amplification and separation of SNP in a PCR instrument wherein the SBE primers provide customizied amplicon lengths to aid electrophoretic

separation. In another important aspect of the present invention, kits are provided for detecting fluoroquinolones resistant *B. anthracis* by thermal recycling amplification, preferably multiplexing. Preferred kits for the detection of fluoroquinoline resistance in *Bacillus anthracis* comprise one or more pairs of oligonucleotide primers pairs selected from the group of oligonucleotide pairs consisting of: SEQ ID NO: 1 and SEQ ID NO: 2; SEQ ID NO:3 and SEQ ID NO: 4; SEQ ID NO: 5 and SEQ ID NO: 6; SEQ ID NO: 7 and SEQ ID NO: 8; SEQ ID NO: 9 and SEQ ID NO: 10; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; SEQ ID NO: 15 and SEQ ID NO: 16; SEQ ID NO: 17 and SEQ ID NO: 18; SEQ ID NO: 19 and SEQ ID NO: 20; SEQ ID NO: 21 and SEQ ID NO: 22; SEQ ID NO: 23 and SEQ ID NO: 24; SEQ ID NO: 25 and SEQ ID NO: 26; SEQ ID NO: 27 and SEQ ID NO: 28; SEQ ID NO: 29 and SEQ ID NO: 30; SEQ ID NO: 31 and SEQ ID NO: 32; SEQ ID NO: 33 and SEQ ID NO: 34; SEQ ID NO: 35 and SEQ ID NO: 36; SEQ ID NO: 37 and SEQ ID NO: 38; SEQ ID NO: 39 and SEQ ID NO: 40; wherein the pair of oligonucleotide primers is capable of causing amplification of a product that indicates fluoroquinoline resistance in *B. anthracis*.

[00020] Most preferably the kits of the present invention further comprise tnps, most preferably labeled, for example, ATP, TTP, GTP, CTP and UTP, and taq polymerase, salts and buffer suitable for causing amplification of said DNA in a PCR instrument. Certain other kits comprise one or more oligonucleotide primer selected from the group consisting of: SEQ ID NO: 41; SEQ ID NO: 42; SEQ ID NO:43; SEQ ID NO: 44; SEQ ID NO: 45; SEQ ID NO: 46; SEQ ID NO: 47; SEQ ID NO: 48; SEQ ID NO: 49; SEQ ID NO: 50; SEQ ID NO: 51; SEQ ID NO: 52; and SEQ ID NO: 53, wherein the primers are capable of detecting a single nucleotide polymorphism, wherein the single nucleotide polymorphism is characteristic of fluoroquinoline

resistance in *Bacillus anthracis*. Most preferably the primers are labeled with an observable marker, a fluorescent or radioactive group. In preferred embodiments, the kits further comprise salts and buffer suitable for causing binding of DNA and primers.

BRIEF DESCRIPTION OF THE DRAWINGS

[00021] FIG. 1 illustrates mutant sensitivity to different Ciprofloxacin concentrations. The wild type progenitor strain (A) and three sequential stepwise mutant strains (B-D) are evaluated for Ciprofloxacin sensitivity using E-strips. The stepwise mutant strains are (B) S1-1, (C) S2-3 and (D) S3- 1. The spontaneous stepwise mutation rates in changes per generation shown in the arrows.

[00022] FIG. 2 illustrates SNP assays for mutational changes associated with CIP resistance. An illustration of an ABI377 gel image is shown with nine SNP loci across seven *B. anthracis* strains (wild type, two step 1 mutants, two step 2 mutants, and two step 3 mutants). In addition, one electropherogram of the wild type genotype generated on a capillary electrophoresis instrument (AB3100) is shown to illustrate the assay's flexibility across diagnostic platforms. SNP of mutant genotypes are: S1-1: *gyrA*254(R) C→T; S1-2: *gyrA*265(R) G→A; S2-1: *gyrA*254(R) C→T & *parC*242 C→T; S2-2: *gyrA*254(R) C→T & *parC*242 C→A; S3-1: *gyrA*254(R) C→T & →265(R) G→A & *parC*242 C→T; S3-2: *gyrA*254(R) C→T & *gyrA*266(R) A→C & *parC*242 C→T.

DETAILED DESCRIPTION

[00023] The present invention discloses a molecular assay for screening *B. anthracis* for single nucleotide polymorphism (SNPs) associated with Ciprofloxacin (CIP) resistance. This diagnostic approach provides a rapid screening of *B. anthracis*

samples for CIP resistence in situations where culturing the sample is not possible. It is anticipated that in the event of any bioterrorist activity, this rapid assay will make possible the early detection of malicious spread of anthrax.

[00024] A study of multiple mutant B anthracis strains showed that the primary target of CIP in wild-type *B. anthracis* is GyrA, the secondary target is ParC and the tertiary targets are yet to be fully determined. The target order of CIP appears to be determined by the amino acid residues of the Gyrase and Toposiomerase IV subunit QRDRs.

[00025] Assays are presented for determining fluoroquinolones resistant *B. anthracis* based on the mutational status of the six *gyrA* and three *parC* nucleotides. Primers for multiplexing to amplify nine loci are disclosed in Table 3 and Figure 2. These nine loci represent the most common mutants and may be quickly assayed using the present methods. Because these nine mutations play a critically important role in determining the level of CIP resistance, SNP information may be used in developing an appropriate antibiotic treatment strategy at an early stage of an outbreak. A newly acquired strain could be genotyped in just a few hours.

[00026] Strains of CIP resistant *B. anthracis* that arise either by misuse of antibiotics or malevolence, can be rapidly genotyped using the disclosed SNP assay This assay was developed using the SNaPshot™ technology (ABI PRISM™ Applied BioSystems Inc.) but other SNP assays known in the art are available and may be used. In the present assay, the mutational status of the six *gyrA* and three *parC* nucleotides is easily observed in a single lane on an ABI377 or AB3100.

[00027] The following definitions are used herein:

[00028] "Polymerase chain reaction" or "PCR" a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to

amplify the number of copies of a target DNA sequence by approximately 106 times or more. The polymerase chain reaction process for amplifying nucleic acid is disclosed in US Pat. Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference.

[00029] "Primer" a single-stranded oligonucleotide or DNA fragment which hybridizes with a DNA strand of a locus in such a manner that the 3' terminus of the primer may act as a site of polymerization using a DNA polymerase enzyme.

[00030] "Primer pair" two primers including, primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

[00031] "Primer site": the area of the target DNA to which a primer hybridizes.

[00032] "Multiplexing" is an assay system for simultaneous, multiple determinations in a single assay process in a thermocycling instrument (PCR). A process to implement such a capability in a process is a "multiplexed assay." Systems containing several loci are called *multiplex* systems described, for example, in US Patent No. 6,479,235 to Schumm, et al., US Patent No. 6,270,973 to Lewis, et al. and 6,449,562 to Chandler, et al.

[00033] "Isolated nucleic acid" is a nucleic acid which may or may not be identical to that of a naturally occurring nucleic acid. When "isolated nucleic acid" is used to describe a primer, the nucleic acid is not identical to the structure of a naturally occurring nucleic acid spanning at least the length of a gene. The primers herein have been designed to bind to both sequences flanking. Certain primers also may bind internally to the DNA sequence of interest. It is to be understood that primer sequences containing insertions or deletions in these disclosed sequences that

do not impair the binding of the primers to these flanking sequences are also intended to be incorporated into the present invention.

Method for rapid assay of *B. anthracis* for detection of fluoroquinolones-reistant strains.

[00034] DNA extraction. DNA was extracted as described in Keim *et. al.*, 2000. Briefly, DNA was extracted from each resistant mutant by suspending ~ 1 mg of cellular material from blood agar plates in 150 µl of heat-soak buffer (10 mM Tris-HC1, 1mM EDTA, pH 8.0) and heating to 85°C for 30 min. Cellular debris was pelleted by centrifugation and the supernatant was used as template in PCR reactions.

[00035] PCR amplification. All primers (Table 1) were designed from the incomplete *B. anthracis* genome sequence generally provided by The Institute for Genomic Research, Rockville, MD, USA. PCR products were amplified in 50 µl PCR reactions and prepared as follows:. 1X PCR buffer (20 mM Tris pH 8.4, 50 mM KCI) (Gibco/BRL, Bethesda, MD, USA), 0.10 mM DNTPs, 2 mM $MgCI_2$, 2 µl heat-soak supernatant as template, 0.04 U/µl *Taq* DNA Polymerase (Gibco/BRL, Bethesda, MD, USA), 0.2 µM forward and reverse primers, adjusted to 50 µl with filtered (0.2 µm) 17.8 mOhm E-pure water. Reactions were heated to 94°C for 5 min, then subjected to 35 cycles of 20 s at 94°C, 20 s at 60°C and 20 s at 72°C. This was followed by heating to 72°C for 5 min to complete primer extension. PCR products were quantified on. EtBr stained 1.5% Synergel™ (Diversified Biotech, Boston, MA, USA)/0.7% agarose (Gibco/BRL, Bethesda, MD, USA). Quantified PCR products were sequenced as follows:.

[00036] DNA sequencing. PCR products were diluted 1:5 in water and sequenced on an ABI377 fluorescent sequencer using the ABI PRISM® Ready Reaction BigDye™ Terminator Cycle Sequencing Kit (both from Perkin-Elmer/Applied

Biosystems Inc., Foster City, CA, USA). When necessary, contiguous gene sequences were prepared from the individual sequences using SeqMan™ software (DNASTAR, inc., Madison, WI, USA). Contiguous Sequences were aligned with the wild-type sequences using MegAlign™ software (DNASTAR, inc., Madison, WI, USA).

[00037] **SNP multiplex assay.** A single nucleotide polymorphism (SNP) assay was developed to rapidly identify the nine observed resistance mutations (Table 2). Flanking primers (Table 3) were designed to amplify bases 203-323 of *gyrA* (122 bp product) and bases 175-320 of *parC* (146 bp product). PCR products were amplified in 10 μl singleplex or duplex PCRs with final concentrations of lx PCR Buffer (above), 3mM $MgC1_2$, 0.1 mM dNTPs, forward and reverse primer pairs (0.1 μM *gyrA* primers / 0.4 μM *parC* primers), 1 U Platinum® *Taq* DNA Polymerase (Gibco/BRL, Bethesda, MD, USA), and 1 μl heat-soak supernatant as template. Reactions were heated to 94°C for 5 min, then subjected to 30 cycles of 20 s at 94°C, 30 s at 60°C and 30 s at 72°C. The remainder of the procedure was carried out according to methods known in the art. Preferred instructions are given in the ABI PRISM® SNaPshot™ Multiplex Kit and run on an AB3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Single base extension (SBE) primers (Table 3) were designed with polynucleotide tails (poly-Cs and single As) to customize amplicon lengths to 4-bp intervals such that when separated electrophoretically, the six *gyrA* SNPs were detected in the 5' to 3' order in which mutations are found, followed by the three *parC* SNPs (also in 5' to 3' order of occurrence). Since primers *gyrA*265 and *gyrA*254 overlapped 1 and 2 SNP loci respectively, they were designed with degenerate base pairs at sites 266 and 265 to allow annealing on templates with step 3 mutations. Despite primer degeneracy, amplification of *gyrA*265 in the 13-

primer multiplex was weak on S3-2 mutants. This locus was therefore targeted individually by performing a second SBE containing only the two *gyrA265* primers when a template was suspected to be an S3-2 mutant. The order of SBE products enabled multiplexing of SBE PCRs and facilitated scoring, eliminating the need for a size standard. Therefore, this assay can be performed on a 4-dye ABI377 if a 5-dye capillary machine is not available.

Table 1. Primers Used

SEQ ID #	Name	Sequence (5'→3')	Gene/Region
SEQ ID #1	ParC QRDR F	GTGTTAGGTGACCGCTTTGCACGTTAT AGTAAATA	parC/QRDR
SEQ ID #2	ParC QRDR R	GTAAAACAACCGGTTCTTCACTCGTAT CATC	parC/QRDR
SEQ ID #3	GyraA QRDR F	ACGTATTAATTCCATAGAGATTTTAGA CATTCTTGCTTCTGTATA	gyrA/QRDR
SEQ ID #4	GyraA QRDR R	CATTTTTAGATTACGCAATGAGTGTTAT CGTATCTCG	gyrA/QRDR
SEQ ID #5	BA ParC aF1	GGTACGACAGTTGCCCAAAATGATGGT T	parC
SEQ ID #6	BA ParC aR1	CAAGCGGAAGCAATTGTATCCT	parC
SEQ ID #7	BA ParC bF1	CGCGTCGATCATCACTATATGTTTTCTT AACTCTC	parC
SEQ ID #8	BA ParC bR1	ATTATTATTCGCGGGAAAGCAGAGGTT GA	parC
SEQ ID #9	BA ParC cF1	GTCTCATCACGTACTTCAGCAATGCCA TCT	parC
SEQ ID #10	BA ParC cR1	TCGGCTAAAACAGTCGGTAACGTTATT GGTAA	parC
SEQ ID #11	BA ParC-E F1	CGGATCCCCGTCAACAC	parC & parE
SEQ ID #12	BA ParC-E R1	CGGATCAATTATGGGAAACAACGATG AATC	parC & parE
SEQ ID #13	BA ParE aF1	AAGCGGGAGGTCATGAAACTTCTCTGC	parE
SEQ ID #14	BA ParE aR1	AGTGGTAAGTTAACACCCGCACAATCA CG	parE
SEQ ID #15	BA ParE bF1	CCCTTGTTTCGCAGAACCAC	parE
SEQ ID #16	BA ParE bRa	TTGAAGCTTTCGTTTCCTAT	parE
SEQ ID #17	BA ParE cF1	CTAATTCTGCTTCAATCCCATTTTGTTC ACC	parE
SEQ ID #18	BA ParE cRa	RAGCGTTATAGATAAAGGGCGAGGAA TG	parE
SEQ ID #19	BA ParE dF1	ACACCGCCATTTTCAAAGCGTTGTTC	parE
SEQ ID #20	BA ParE dR1	GATTTTGGATTAGGAAAGGGGCAAGG AGTT	parE
SEQ ID #21	BA GyrB aF1	CGACGGAATTGAACACGAAACA	gyrB
SEQ ID #22	BA GyrB aR1	TACAGATGCCCCAACACC	gyrB
SEQ ID #23	BA GyrB bF1	ATGGGACGTCCTGCTGTAGAAGTTATT ATGACC	gyrB
SEQ ID #24	BA GyrB bR1	AGTTAAACCTTCACGAACGTCCTCACC AGTTA	gyrB
SEQ ID #25	BA GyrB cF1	ACGTATGAAGGTGGAACACATGAAGT	gyrB

SEQ ID #	Name	Sequence (5'→3')	Gene/Region
		AGGGTTTA	
SEQ ID #26	BA GyrB cR1	GCTTTCTCAATATCAAAATCTCCGCCA ATGT	gyrB
SEQ ID #27	BA GyrB dF1	CGTCACTTCCAAGCGATTTTACCACTG AA	gyrB
SEQ ID #28	BA GyrB dR1	ACCTCCTCTTACATTTCCGTTACACATA CATTGATTTAT	gyrB
SEQ ID #29	BA GyrB-A F1	GGGGGATAAAGTAGAGCCACGTCGTA ACT	gyrB & gyrA
SEQ ID #30	BA GyrB-A R1	AGGAAAACGCGCTGGTAACA	gyrB & gyrA
SEQ ID #31	BA GyrA aF1	CAGCAATGCGTTATACAGAAGCAAGA ATGTC	gyrA
SEQ ID #32	BA Gyra aR1	TGCCTTTTCAAGTTCATAAGCAGTA	gyrA
SEQ ID #33	BA GyrA bF1	GGAAGTACGTCGTGATGCCAATGCTAA TG	gyrA
SEQ ID #34	BA GyrA bR1	ATACCTTTCGCTGTACGACTATACTCTG GGATTTC	gyrA
SEQ ID #35	BA GyrA cF1	CAGAACAAAACATCGCCATTACGTTAA CTCATAA	gyrA
SEQ ID #36	BA GyrA cR1	AGAGATTTGATCAACTGGCATACGAAT AATAACACC	gyrA

Table 2. Identity of Ciprofloccion Resistant Signatures

Mutant	MIC [CIP μg /ml]	*gyrA* mutation		parC mutation	
		Δ Nucleotide	Δ Amino Acid	Δ Nucleotide	Δ Amino Acid
S1-1	0.38	C254→T	S85→L	-	-
S1-2	0,38	G265→A	E89→K	-	-
S1-3	≥0.25	G248→A	G83→D	-	-
S1-4	≥0.25	G250→A	D84→N	-	-
S1-5	≥0.25	G247→T	G83→C	-	-
S2-1	12	C254→T	S85→L	C242→T	S81→F
S2-2	4	C254→T	S85→L	C242→A	S81→Y
S2-3	≥1.5	C254→T	S85→L	G253→A	E85→K
S2-4	≥1.5	C254→T	S85→L	A254→G	E85→G
S3-1	64	C254→T	S85→L	C242→T	S81→F
		G265→A	E89→K		
S3-2	64	C254→T	S85→L	C242→T	S81→F
		A266→C	E89→A		
S3-x	16, 24, 48, 64	C254→T	S85→L	C242→T	S81→F

Table 3. External and SBE primers used in the SNP assay

SEQ ID #	Name	Sequence (5'→3')	Product Size	SNP
	External primers			
SEQ ID #37	BA*gyrA*01F_flanking	TCAGCACGTATTGTTGGTGAAG	122	-
SEQ ID #38	BA*gyrA*01R_flanking	TGCCCATCAACAAGCATATAAC	122	-
SEQ ID #39	BA*parC*02F_flanking	AAAGCGTTCCGTAAGTCGG	145	-
SEQ ID #40	BA*parC*02R_flanking	TTATTACCATGCATCTCAACTAAAAC	145	-
	SBE primers			
SEQ ID #41	BA*gyrA*SNP247F_internal	ATCGGTAAGTATCACCCTCAT	22	G/T
SEQ ID #42	BA*gyrA*SNP248F_internal	CccccCGGTAAGTATCACCCTCATG	26	G/A
SEQ ID #43	BA*gyrA*SNP250F_internal	cccccccGGTAAGTATCACCCTCATGGT	30	G/A
SEQ ID #44	BA*gyrA*SNP254R_internal	cccccccccccccCCATCGTTTCATAAACAGCT	34	C/T†
SEQ ID #45	BA*gyrA*SNP254R(G)_internal	cccccccccccccCCATCGTTGCATAAACAGCT	34	C/T†
SEQ ID #46	BA*gyrA*SNP254R(T)_internal	cccccccccccccCCATCGTTTTATAAACAGCT	34	C/T†
SEQ ID #47	BA*gyrA*SNP254R(GT)_internal	cccccccccccccCCATCGTTGTATAAACAGCT	34	C/T†
SEQ ID #48	BA*gyrA*SNP265R_internal	ccccccccccccccccccGCCATACGTACCATCGTTT	38	G/A†
SEQ ID #49	BA*gyrA*SNP265R(G)_internal	ccccccccccccccccccGCCATACGTACCATCGTTG	38	G/A†
SEQ ID #50	BA*gyrA*SNP266R_internal	cccccccccccccccccccccccCGCCATACGTACCATCGTT	42	A/C†
SEQ ID #51	BA*parC*SNP242F_internal	cccccccccccccccccccccccccccccccCACCCGCACGGTGATT	47	C/T/A
SEQ ID #52	BA*parC*SNP253R_internal	cccccccccccccccccccccccccccccGACTTAAACGTACCATCGCTT	51	G/A†
SEQ ID #53	BA*parC*SNP254R_internal	ccccccccccccccccccccccccccccccccCAGCGATGGTACGTTTAAGTC	54	A/G†

†SNPs will be detected as reverse complements in the SNaPshot™ assay when reverse SBE primers are used.

Selection of mutant *B. anthracis* strains and identification of fluoroquinolones-resistant sites

[00038] **Bacterial strains.** Selections were performed on the non-virulent, pX01-/ pX02-, Ames strain of *B. anthracis* (*Ivins et al.*, 1986). All DNA samples used for the

diversity study came from our *B. anthracis* DNA collection (*Keim, et al.*, 2000). *B. anthrocis* strains were selected sequentially at increasing CIP concentrations to produce a resulting stepwise accumulation of mutations. Mutant strains were isolated with MICs as high as 64μg CIP/ml (1000-fold higher than wild-type) These results are given in Table 2. The accumulation of mutations occurred in a distinctive and ordered manner. First level mutants, selected on 0.25 μg CIP/ml, developed at a rate of 6.6 x 10^{-10} and had one of five mutations within the *gyrA* QRDR (Table 2). A disproportionate number (71%) of these mutants possessed the C254→T missense mutation in *gyrA* (Table 2). The level of resistance conferred by this mutation was similar to that of other S1 mutations. Since this mutation provided no selective advantage over the other S1 mutations, it is reasonable to call the C254 nucleotide of *B. anthracis gyrA* a mutational hotspot. Second level mutants, selected on 1.5 μg CIP/ml, developed at a rate of 1.0 x 10^{-8} and possessed one of four mutations within the *parC* QRDR (Table 2). As with the S1 mutants one S2 genotype, C242→T, was overrepresented (71%) (Table 2). While it is likely that the C242 nuelcotide of *parC* represents another mutational hotspot, the overrepresentation could also be a result of the disproportionate level of resistance conferred to the strain by the mutation (Table 2). Third level mutants, selected on 24 μg CIP/ml, developed at a rate of 4.8 x 10^{-10}. Two third-level mutants were identified with novel mutations within the *gyrA* QRDR (Table 2). However, the other 21 third-level mutants had no additional alterations in either the *gyrA* or *parC* QRDRs. Potential QRDRs in *gyrB* and *parE* were also sequenced from these strains and revealed no additional mutations in these regions. The targeted stepwise accumulation of mutations (Sl *gyrA* → S2 *parC* → S3 *gyrA/?*) give further evidence to support the hypothesis that particular fluoroquinolones have

different primary topoisomerase targets within various bacterial species (Ferrero *et al.*, 1995; Ng *et al.*, 1996; Pan and Fisher *et al.*, 1998).

[00039] *B. anthracis* has the ability to develop a number of different missense mutations that enable it to grow in the presence of CIP. The stepwise phenotypic rates at which *B. anthracis* develops resistance to CIP (4.8×10^{-10} to 1.0×10^{-8}) are similar to those reported for fluoroquinolone resistance in other species. The rarity of human anthrax cases and the carcass-dependent transmission cycle of this pathogen make the development and spread of CIP resistant *B. anthracis* through patient non-compliance unlikely. However, the agricultural practice of antimicrobial growth promotion does have this potential outcome. CIP regimens targeted at serum and tissue concentrations of ≥0.38 μg CIP/ml would reduce the chances for developing CIP resistant *B. anthracis* by requiring the statistically unlikely event (6.6×10^{-18}) of a bacterium to develop advantageous mutations in the *gyrA* and *parC* QRDRs simultaneously. The serum and tissue concentrations resulting from the low-level feeding of antibiotics as antimicrobial growth promoters in livestock would not reach the MPC and likely fall short of the MIC for wild-type *B. anthracis*. Therefore, this practice could present a potential risk for the development of resistant strains, particularly in those livestock regions to which *B. anthracis* is endemic.

[00040] **Diversity Study.** The QRDRs of *gyrA* and *parC* were sequenced from eight major diversity groups and analyzed for point mutations as described below. The eight strains, 3 (74-42C-8), 25 (14185), 39 (46), 45 (2B80), 62 (Oct-321), 77 (Vollum), 80

[00041] **Stepwise mutant selection.** *B. anthracis* Ames -/- strain was taken from a frozen stock, streaked onto blood agar plates and grown overnight at 35°C. Cells from isolated colonies were used to inoculate culture tubes containing 5 ml of

Mueller-Hinton broth. Cultures were incubated overnight at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) shaking at 225 rpm. Each of these cultures (mean OD_{625} ~1.4 or 1.43 x 10^8 CFU/ml) was transferred to a 0.45 μm nitrocellulose membrane filter (Millipore, Bedford, MA USA). Membranes were placed cell-side-up onto Mueller-Hinton agar containing 0.25 μg CIP/ml and incubated for ~40 h. Cells from a single colony from each positive plate were streaked onto blood agar and grown overnight at 35°C. Cells from these plates were used to prepare frozen stocks and to isolate DNA for sequencing (see below). The most common unique genotype, S1-1, was subjected to a subsequent round of selection on Mueller-Hinton agar containing 1.5 μg CIP/ml. Likewise, the most common genotype from this selection, S2-1, was subjected to a third and final selection on agar containing 24 μg CIP/ml.

[00042] **Mutation rates.** Mutation rates for steps 1, 2 and 3 ciprofloxacin resistant mutants were determined using 96 independent cultures of the wild type Ames -/-, S1-1, and S2-1, respectively. A single colony of the starting isolate was suspended in LB broth and used to inoculate each of the independent cultures with approximately 1,000 cells. For steps 1 and 3 mutants, 96 1 ml cultures were grown in LB broth in four 24-well plates (Costar). For step 2, 96 100 μI were grown in LB broth cultures in a single 96-well plate (Costar). All plates were incubated overnight at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) shaking at 225 rpm. Six cultures were chosen at random for each step and used to determine the average total number of cells present in each culture. The remaining 90 cultures were plated onto Mueller-Hinton ciprofloxacin plates with concentrations of 0.25 μg CIP/ml, 1.5 μg CIP/ml, and 24 μg CIP/ml for steps 1, 2, and 3, respectively. For step 2, the 100 μl cultures were directly plated. For steps 1 and 3, the 1 ml

cultures were transferred to sterile 1.5 ml microcentrifuge tubes and centrifuged at 3,000 x g for 5 min. Approximately 850 µl of the supernatant was removed, the pellet was resuspended in the remaining broth and plated. All of the plates were incubated at 37°C for ~48 h. Up to four putative resistant colonies from each positive plate were transferred to fresh selective medium, and incubated at 37°C for ~48 h to confirm resistance. The number of plates devoid of resistant mutants represents zero mutational events. This value was used with the cell count in the Poisson distribution to estimate the mutation rate for each step.

[00043] **Susceptibility testing.** MICs were determined by the Mueller-Hinton agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (National committee, 1997). The E-test strips (AB BIODISK) were used for rapid screening and are shown in Figure 1.

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[00044] While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.